Revisiting the roles of hepatic inflammation and adipokines in metabolic disease

Gruben, Nanda

Chapter 4

Determining the association between adipokine expression in multiple tissues and phenotypic features of nonalcoholic fatty liver disease in obesity

Marcel G.M. Wolfs[1,*], Nanda Gruben[1,*], Sander S. Rensen[2], Froukje J. Verdam[2], Jan Willem Greve[2], Ann Driessen[3,4], Cisca Wijmenga[5], Wim A. Buurman[6], Lude Franke[5,7], Ludger Scheja[8], Debby P.Y. Koonen[1], Ronit Shiri-Sverdlov[9], Timon W. van Haeften[1,10], Marten H. Hofker[1,*] and Jingyuan Fu[5,*]

[1]University of Groningen, University Medical Center Groningen, Department of Pediatrics, Molecular Genetics Section, Groningen, the Netherlands; [2]Maastricht University Medical Center, NUTRIM School for Nutrition, Toxicology and Metabolism, Department of General Surgery, Maastricht, the Netherlands; [3]Maastricht University Medical Centre, NUTRIM School for Nutrition, Toxicology and Metabolism, Department of Pathology, Maastricht, the Netherlands; [4]Department of Pathology, University Hospital Antwerp, Edegem, Belgium; [5] University of Groningen, University Medical Center Groningen, Department of Genetics, Groningen, the Netherlands; [6]School for Mental Health and Neuroscience, Maastricht University, Maastricht, The Netherlands; [7]Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry, London, UK; [8]University Medical Center Hamburg-Eppendorf, Department of Biochemistry and Molecular Cell Biology, Hamburg, Germany; [9]Maastricht University, Department of Molecular Genetics, Maastricht, the Netherlands; [10]University Medical Center Utrecht, Department of Internal Medicine, Utrecht, the Netherlands

[*] Contributed equally

Nutrition and Diabetes 2015, 5: e146. doi: 10.1038/nutd.2014.43

Abstract

Nonalcoholic fatty liver disease (NAFLD) is an obesity-associated disease and in obesity, adipokines are believed to be involved in the development of NAFLD. However, it is still not clear whether adipokines in liver and/or adipose tissues can be related to the development of specific characteristics of NAFLD, such as steatosis and inflammation. We aimed to address this question by simultaneously examining the adipokine expression in three tissue types in obese individuals. We enrolled 93 severely obese individuals with NAFLD, varying from simple steatosis to severe nonalcoholic steatohepatitis (NASH). Their expression of 48 adipokines in liver, visceral and subcutaneous adipose tissue was correlated to their phenotypic features of NAFLD. We further determined whether the correlations were tissue-specific and/or independent of covariates, including age, sex, obesity, insulin resistance and type 2 diabetes. The expression of adipokines showed a liver and adipose tissue-specific pattern. We identified that the expression of *leptin*, *ANGTP2* and *chemerin* in visceral adipose tissue was associated with different NAFLD features, including steatosis, ballooning, portal and lobular inflammation. In addition, the expression of *TNF*, *PAI-1*, *IGF1*, *CXCL10* in liver tissue and the expression of *IL1RN* in both liver and subcutaneous adipose tissue were associated with NAFLD features. The correlations between *ANGPT2* and *CXCL10* and NAFLD features were dependent on insulin resistance and type 2 diabetes, but for the other genes the correlation with at least one NAFLD feature remained significant after correcting for the covariates. Our results suggest that in obese individuals, visceral adipose tissue-derived *leptin* and *chemerin* and hepatic expression of *TNF, IGF1, IL1RN* and *PAI-1* are involved in the development of NAFLD features. Further functional studies are warranted to establish a causal relationship.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is an obesity-associated disease ranging from relatively benign hepatic steatosis, to nonalcoholic steatohepatitis (NASH), severe cirrhosis and fibrosis. NASH is characterized by steatosis, hepatic inflammation, and cytological ballooning [1]. With the increasing prevalence and severity of obesity and type 2 diabetes, more patients with NASH will progress to potentially fatal, end-stage liver disease. Specifically, it was shown that almost half of the patients with NASH have advanced fibrosis [2] and that 10 to 25% of the NASH patients will progress to show severe liver pathology such as liver cirrhosis and/or hepatocellular carcinoma [3-6], which are associated with high morbidity and mortality [3,5,7].

Since NAFLD is strongly associated with obesity and the metabolic syndrome [8], one hypothesis is that the altered release of adipokines (signaling molecules from the adipose tissue) that occurs in the metabolic syndrome may be involved in the development of NAFLD [9]. These adipokines can enter the bloodstream and reach the liver [10]. Adipokines derived from visceral adipose tissue (VAT) may be specifically important, since visceral adiposity is related to obesity-associated co-morbidities such as NAFLD [11]. Previous studies have identified an association between elevated plasma levels of the adipokine leptin and NASH in humans [12]. However, since many adipokines are also expressed in the liver and other organs [13,14], a link between peripheral plasma levels and NASH does not provide evidence that the expression of these adipokines in VAT is associated with NASH.

To date, 48 adipokines have been defined in the literature [15-18]. However, no systematic analyses have been reported on their role in the development of specific characteristics of NAFLD, such as steatosis and inflammation. In this study, we correlated the expression of 48 adipokines in human hepatic, visceral and subcutaneous adipose tissue (SAT) to specific features of NAFLD in severely obese individuals. We aimed to identify if adipokines are associated to certain phenotypic features of NAFLD.

Subjects and methods

Study population

Between 2006 and 2009, 93 severely obese individuals with a body mass index (BMI) ranging from 30–74 kg/m^2 underwent elective bariatric surgery at the Department of

General Surgery, Maastricht University Medical Centre (Maastricht, the Netherlands). Subjects with acute or chronic inflammatory diseases, degenerative diseases, and those reporting an alcohol intake exceeding 10 g/day or using anti-inflammatory drugs were excluded. This study was approved by the Medical Ethics Board of Maastricht University Medical Centre and informed written consent was obtained from each individual.

Histological assessment of liver pathology

Wedge liver biopsies were fixed in formalin and embedded in paraffin for histological staining. They were analyzed by an experienced pathologist who was blinded to the clinical and biochemical parameters. Each individual was scored for seven different histological parameters of liver pathology: steatosis, fibrosis, inflammation (lobular inflammation, large lipogranulomas, portal inflammation), liver cell injury (ballooning) and glycogenated nuclei according to the scoring system described by Kleiner *et al.* [19].

Tissue sampling, histology preparation, and mRNA isolation

Tissue sampling and RNA isolation have been described before [20]. In brief, RNA was isolated from VAT, SAT and liver tissue obtained during bariatric surgery with the Qiagen Lipid Tissue Mini Kit (Qiagen, Hilden, Germany, 74804). The Agilent Bioanalyzer (Agilent Technologies, Waldbronn, Germany, 5067-1521) was used to assess RNA quality and concentration.

mRNA profiling and normalization

mRNA pre-hybridization processing and hybridization were performed as described before [20,21]. Anti-sense RNA synthesis, amplification and purification were performed according to the manufacturer's protocol, using the Ambion Illumina TotalPrep Amplification Kit (Applied Biosystems/Ambion, Austin, TX, USA). Complementary RNA was hybridized to microarrays containing 48,755 probes, which targeted 37,776 different genes (Illumina HumanHT12 BeadChips, Illumina, San Diego, CA, USA). These microarrays were scanned on the Illumina BeadArray Reader. After quality control and normalization [20], a total of 82 liver samples, 90 SAT samples, and 84 VAT samples were retained for further analysis. The expression data are freely available in the Gene Expression Omnibus (GSE22070).

mRNA analysis of adipokines

To gain insight into the tissue-specific expression of adipokines and their roles in NAFLD, we focused on 69 probes that targeted 48 adipokines, which have been well defined in the literature [15-18]. If multiple probes were annotated for the same gene, we took the average intensity of these probes per gene. All analyses were done using R (version 2.15.1) (R foundation for statistical computing, Vienna, Austria). The heatmap and hierarchical clustering of adipokine expression in these three tissue samples was plotted using R package Gplots (version 2.11.0). The distance was calculated using the Euclidean distance, and the samples and genes were clustered using the "complete" method. To identify potentially confounding factors, we calculated Spearman correlation coefficients between phenotypic features of NAFLD and sex, age, BMI, waist-to-hip ratio (WHR), the diagnosis of type 2 diabetes (T2D), and the homeostasis model assessment of insulin resistance (HOMA-IR). In addition, the inter-correlation among NAFLD features and the inter-tissue correlation of adipokine expression levels were computed using the same method (see Supplementary Statistical Notes 1).

To determine how adipokines are involved in NAFLD, we first computed Spearman correlations between liver histology scores and the expression levels of adipokines from each tissue type. The significance of correlation was determined as 7.03×10^{-4}, which corresponds to a false discovery rate (FDR) of 0.05, using 1000x permutations (see Supplementary Statistical Notes 2). A partial correlation analysis was performed to correct for inter-tissue correlations of adipokine expression levels and to determine the most relevant tissue type (R package ppcor, version 1.0). To regress out the effect of confounding factors (age, sex, BMI, WHR, T2D and HOMA-IR), we performed conditional regression analysis (see Supplementary Statistical Notes 3).

Plasma measurement of chemerin and ANGPT2

For determination of plasma adipokine levels, venous blood samples were collected in EDTA tubes after an 8-hour fasting period. Plasma was used to measure chemerin and angiopoietin 2 (ANGPT2) levels using commercially available ELISAs (R&D systems, Abingdon, UK). A Spearman correlation analysis was used to test the correlation between plasma levels, gene expression, and different NAFLD features.

Results

Severely obese individuals show varying degrees of NAFLD

Plasma parameters and clinical traits of all the subjects are shown in Supplementary Table 1. We scored seven histological characteristics of NAFLD in 93 severely obese individuals, following Kleiner *et al.*'s method [19]. Of these individuals, 25 showed no signs of NAFLD, while eight showed simple steatosis without inflammation. The other 60 individuals had steatosis accompanied by varying degrees of inflammation (Table 1).

TABLE 1. Summary of liver pathology of the study population.

Features	Individuals scored (#)	Grade (# of individuals)			
		0	1	2	3
Steatosis (0-3)	93	25	26	29	13
Fibrosis (0-3)	91	62	15	11	3
Lobular inflammation (0-3)	88	36	35	12	5
Large lipogranulomas (0-1)	92	70	22	-	-
Portal inflammation (0-1)	93	72	21	-	-
Ballooning (0-2)	92	49	39	4	-
Glycogenated nuclei (0-1)	90	74	16	-	-

The data shown are the total number of individuals scored for each marker and the number of individuals with different grades for the seven features of nonalcoholic fatty liver disease, as described in [19].

NAFLD characteristics correlated with other phenotypes and adipokine expression shows inter-tissue correlations

No strong associations between NAFLD features and sex or age were observed (Fig. 1A). In line with clinical observations [8], the NAFLD features steatosis, fibrosis, ballooning and inflammation were correlated with BMI, WHR, T2D and HOMA-IR (Fig. 1A). Many NAFLD features also showed significant inter-correlations. For example, in accordance with its central role in NAFLD, steatosis was correlated with all other NAFLD features scored (Fig. 1B).

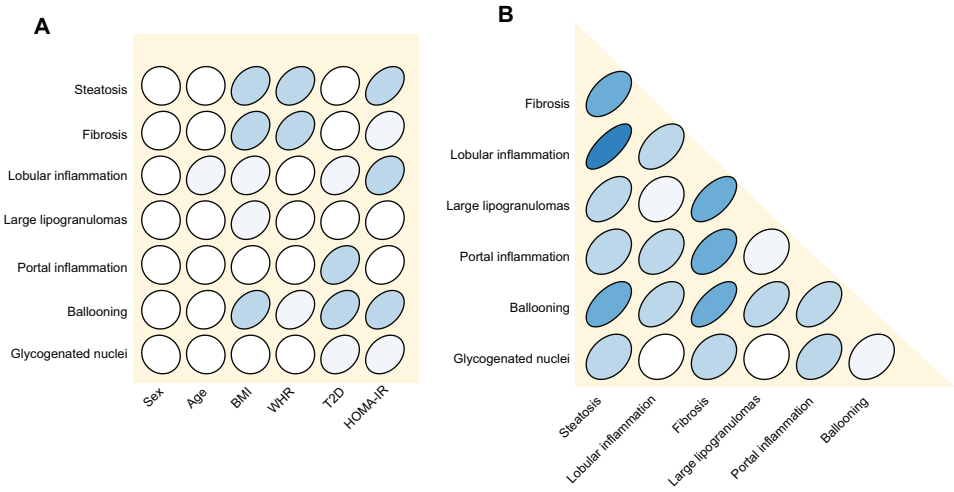


FIGURE 1. Correlations of features of nonalcoholic fatty liver disease. (A) The correlation between the nonalcoholic fatty liver disease (NAFLD) features and sex, age, BMI, WHR, T2D and HOMA-IR. (B) The inter-correlation among the NAFLD features. The correlations were computed with Spearman correlation and confidence ellipses serve as visual indicators of correlation. All correlations significant at $P \leq 0.05$ are indicated in blue. The darker shade of blue indicates a stronger correlation.

Hierarchical clustering of samples based on the genome-wide gene expression in the liver, SAT and VAT (Supplementary Fig. 1) showed that sample clusters did not correspond to any cofactors or NAFLD features. Thus, general differences in gene expression in obese individuals cannot explain a possible association between adipokine expression and NAFLD features (Supplementary Fig. 1).

Most of the adipokines showed tissue-dependent expression, which allowed us to classify tissue samples based on their expression profile (Fig. 2A). Of the 48 adipokines tested, 26 did not show inter-tissue correlation, but 22 adipokines showed expression levels that were correlated between two or three tissues (Fig. 2B). Twenty adipokines showed an inter-tissue correlation between VAT and SAT, and six adipokines showed an inter-tissue correlation between SAT and hepatic tissue. The expression of ten adipokines was correlated between VAT and hepatic tissue (Fig. 2B).

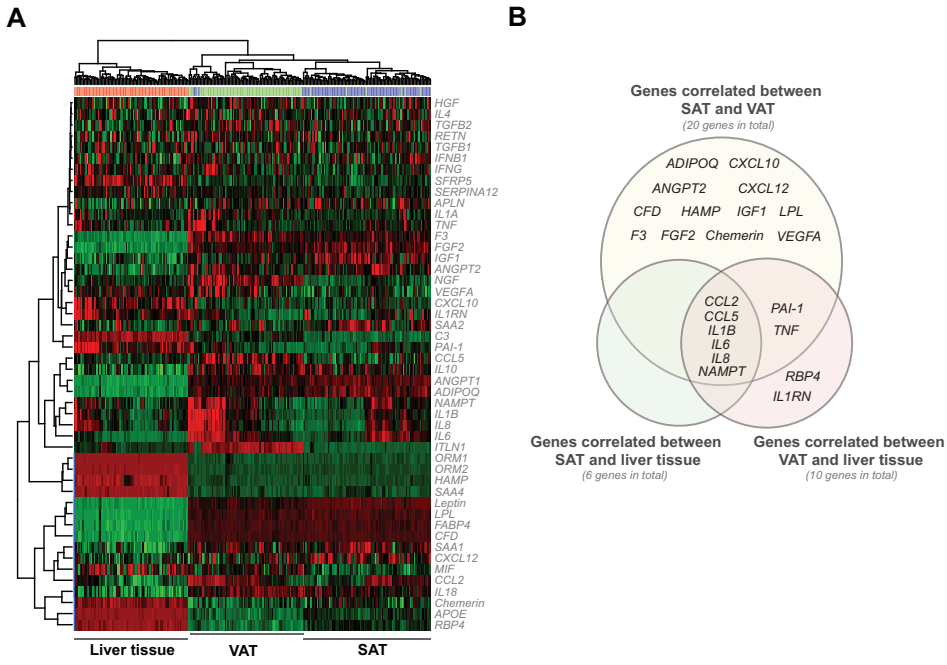


FIGURE 2. The expression of adipokines in liver tissue, subcutaneous and visceral adipose tissue. (A) The heatmap diagram shows the differential gene expression in joints from the three tissue types. The tissue samples (top row: red, liver samples; green, VAT samples; blue, SAT samples) and genes (left side) are clustered hierarchically. Each column represents a tissue sample and each row represents the expression of a single gene (green, low expression; red, high expression). (B) Venn diagram showing correlation of gene expression between tissues at false discovery rate = 0.05.

The expression of eight adipokines was associated with NAFLD features

We observed that the expression levels of eight adipokines (*angiopoietin 2* (*ANGPT2*), *leptin*, *chemerin*, *tumor necrosis factor* (*TNF*), *interleukin* (*IL*)*-1 receptor antagonist* (*IL1RN*), *chemokine* (*C-X-C motif) ligand 10* (*CXCL10*), *insulin-like growth factor 1* (*somatomedin C*) (*IGF1*) and *plasminogen activator inhibitor type 1* (*PAI-1*)) in one or more tissue types correlated with steatosis, ballooning, lobular inflammation, portal inflammation, and/or fibrosis at $P < 7.0 \times 10^{-4}$ (FDR < 0.05) (Table 2, Fig. 3). Large lipogranulomas and glycogenated nuclei did not correlate with adipokine expression levels. Since the expression levels of many adipokines showed inter-tissue correlations (Fig. 2B), we performed a partial correlation analysis to correct for the impact of the other tissues. This allowed us to identify the most relevant tissue type associated to NAFLD. The expression of *chemerin* in both SAT and VAT was negatively correlated with

lobular inflammation. Taking into account the co-expression of *chemerin* in both adipose tissues (Fig. 2B), the partial correlation analysis showed that only *chemerin* expression in VAT was associated with lobular inflammation (Table 2). In this way, we identified three adipokines (*ANGPT2*, *leptin* and *chemerin*) whose expression in VAT was associated to lobular inflammation, ballooning and/or steatosis. In addition, the expression of *TNF*, *IL1RN*, *CXCL10*, *IGF1* and *PAI-1* in the liver, and the expression of *IL1RN* in SAT, were correlated to NAFLD features (Table 2).

Of the individuals in our cohort, 67 out of 93 (72%) were women with an age ranging between 17 and 65 years, including 13 women aged over 50, some of whom might be post-menopausal. The menopausal status may have an effect on cytokine expression and NAFLD development, but we had no information on their menopausal status. We therefore first investigated the correlation between adipokines and NAFLD features by correcting for age and gender. After correcting for this, all the detected correlations remained significant (Table 2). Then we confined the analysis to 54 women under 50 years old and observed the same effects (Supplementary Table 2). These results suggest that the correlations detected between adipokines and NAFLD development are independent of age, sex and menopausal status.

NAFLD is an obesity-related disease and shows high comorbidity with other obesity-related complications, such as insulin resistance and type 2 diabetes. Our data also showed that BMI, WHR, T2D and HOMA-IR were correlated with several NAFLD features (Fig. 1A). We therefore examined whether the correlation between adipokine expression and NAFLD features was dependent on these confounders. After step-wise correction for the effect of age, sex, obesity and diabetes parameters, the correlations between *leptin* expression in VAT and steatosis were no longer significant. In addition, the correlation for *CXCL10* expression in liver and *ANGPT2* expression in VAT disappeared (Table 2), which indicates that these correlations were largely dependent on the obesity and other obesity-related disorders. Nevertheless, the other correlations were still significant at $P < 0.05$ after this correction (Table 2, Fig. 3).

TABLE 2. The adipokines that were correlated with features of nonalcoholic fatty liver disease.

Gene	Feature	Tissue	Not corrected Correlation P-value	Tissue corrected Correlation P-value	Age, Sex corrected Correlation P-value	Age, Sex, obesity corrected Correlation P-value	Age, Sex, Obesity, T2D and IR corrected Correlation P-value
ANGPT2	Lobular inflam.	VAT	0.42 P=1.1x10^{-4}	0.36 P=2.1 x10^{-3}	0.45 P=3.1x-10^{-5}	0.40 P=0.0027	0.24 P=0.11
Leptin	Lobular inflam.	VAT	0.39 P=4.0x10^{-4}	0.34 P=3.3x10^{-3}	0.406 P=2.1x10^{-4}	0.33 P=0.016	0.28 P=0.05
	Steatosis	VAT	0.41 P=9.4x10^{-5}	0.40 P=2.5x10^{-4}	0.454 P=1.46x10^{-5}	0.038 P=0.781	-0.031 P=0.83
Chemerin	Lobular inflam.	VAT	-0.51 P=1.5x10^{-6}	-0.41 P=3.0x10^{-4}	-0.552 P=1.33x10^{-7}	-0.54 P=2.9x10^{-5}	-0.49 P=5.1x10^{-4}
	Lobular inflam.	SAT	-0.37 P=5.6x10^{-4}	-0.17 P=0.17	-0.347 P=0.00114	-0.32 P=0.016	-0.22 P=0.13
	Portal inflam.	VAT	-0.37 P=5.9x10^{-4}	-0.31 P=7.2x10^{-3}	-0.375 P=4.5x10^{-3}	-0.34 P=0.0092	-0.28 P=0.05
	Steatosis	VAT	-0.39 P=2.9x10^{-4}	-0.24 P=0.037	-0.39 P=2.1x10^{-3}	-0.32 P=0.015	-0.31 P=0.03
IL1RN	Lobular inflam.	Liver	0.46 P=2.2x10^{-5}	0.38 P=9.1x10^{-4}	0.44 P=4.9x10^{-5}	0.45 P=4.5x10^{-4}	0.40 P=0.0038
	Ballooning	SAT	0.39 P=1.4x10^{-4}	0.41 P=2.4x10^{-3}	0.38 P=2.6x10^{-4}	0.42 P=9.2x10^{-4}	0.32 P=0.019
CXCL10	Steatosis	Liver	0.38 P=4.9x10^{-4}	0.35 P=1.7x10^{-3}	0.38 P=5.2x10^{-4}	0.27 P=0.036	0.25 P=0.069
	Fibrosis	Liver	0.40 P=2.9x10^{-4}	0.33 P=3.5x10^{-3}	0.40 P=2.0x10^{-4}	0.19 P=0.16	0.21 P=0.145
IGF1	Steatosis	Liver	-0.41 P=1.5x10^{-4}	-0.37 P=9.8x10^{-4}	-0.43 P=4.8x10^{-5}	-0.32 P=0.012	-0.29 P=0.037
PAI-1	Lobular inflam.	Liver	0.55 P=2.5x10^{-7}	0.42 P=2.0x10^{-4}	0.52 P=1.1x10^{-6}	0.49 P=9.4x10^{-5}	0.43 P=0.0018
	Portal Inflam.	Liver	0.37 P=6.6x10^{-4}	0.25 P=0.028	0.38 P=4.8x10^{-4}	0.46 P=1.7x10^{-4}	0.39 P=0.0034
TNF	Lobular Inflam.	Liver	0.38 P=7.0x10^{-4}	0.29 P=0.014	0.34 P=0.0027	0.27 P=0.044	0.38 P=0.0063

The table shows all the adipokines that were correlated with liver histology (FDR < 0.05) and the correlation adjusted for tissue type and covariates, including age, sex, BMI, WHR, HOMA-IR and T2D. The texts with gray color indicate the correlations that were not significant at $P \leq 0.05$ after correction.

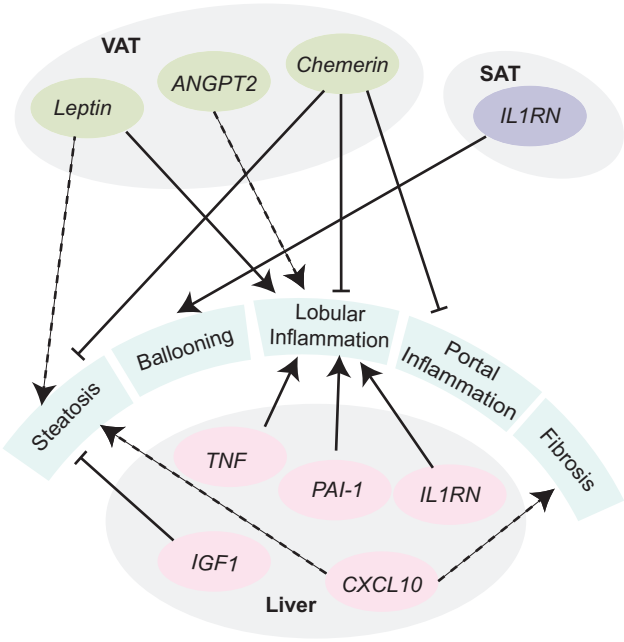


FIGURE 3. The tissue-specific correlation between adipokine expression and liver histology. Each gene node indicates the expression of a gene in a certain tissue type and each line indicates a significant correlation with liver histology after correction for expression in other tissue types. The arrowhead indicates a positive correlation and the bar head a negative correlation ($P < 0.05$). The dashed line indicates a correlation that is dependent on covariates, including age, sex, BMI, WHR, T2D and/or HOMA-IR.

Peripheral plasma chemerin and ANGPT2 levels do not correlate with NAFLD features

Adipokines derived from VAT may be important in the etiology of NAFLD, since visceral adiposity is mainly related to obesity-associated NAFLD [11]. As adipokines expressed in adipose tissue are expected to have endocrine effects on the liver through secretion in the bloodstream, we measured plasma chemerin and ANGPT2 levels (Supplementary Table 1). However, we found no evidence that plasma chemerin and ANGPT2 levels were associated to NAFLD features (Table 3). Moreover, plasma chemerin levels did not correlate with *chemerin* expression in any of the three tissues. Only hepatic expression of *ANGPT2* correlated with plasma ANGPT2 levels (Table 3).

TABLE 3. Correlation between plasma adipokine levels and tissue expression levels or features of nonalcoholic fatty liver disease.

	ANGPT2		Chemerin	
	Correlation	P-value	Correlation	P-value
Expression levels				
Liver	0.313*	0.035	0.171	0.255
SAT	0.225	0.128	0.098	0.511
VAT	0.198	0.198	0.066	0.671
Features				
Steatosis	0.098	0.501	-0.072	0.622
Fibrosis	0.201	0.170	-0.275	0.058
Lobular inflammation	0.028	0.852	-0.118	0.431
Large lipogranulomas	-0.005	0.973	-0.174	0.236
Portal inflammation	0.148	0.309	0.152	0.296
Ballooning	0.239	0.102	-0.184	0.209
Glycogenated nuclei	-0.052	0.730	-0.099	0.509

Correlation coefficients of plasma adipokine levels and tissue expression levels or features of nonalcoholic fatty liver disease are shown, with their respective p-value. The significance threshold for these correlation coefficients was 0.28 (* $p < 0.05$). SAT, subcutaneous adipose tissue; VAT, visceral adipose tissue; ANGPT2, angiopoietin 2.

Discussion

We have simultaneously assessed the expression of 48 adipokines in liver, VAT and SAT to identify which adipokines in which tissues were associated with specific features of NAFLD. We observed that the hepatic expression of five genes, including *TNF*, *IGF1*, *IL1RN*, *PAI-1* and *CXCL10*, was correlated with NAFLD features. These correlations were independent of obesity and T2D, except for *CXCL10*. The central role of these adipokines in the progression of NAFLD is supported by many publications. *TNF* activates c-Jun NH2-terminal Kinase (JNK) and inhibitor of κB-kinase-β (IKKβ), thus resulting in increased production of additional inflammatory cytokines [22]. In addition, enhanced TNF-signaling was reported to aggravate hepatic inflammation and fibrosis in mice [23]. These findings are in line with the positive association we found between *TNF* expression in the liver and lobular inflammation. *IL1RN* is an antagonist of the IL-1 receptor and reduces the inflammation related activities of interleukin 1A and 1B [24]. IL1RN serum levels as well as liver mRNA expression have been correlated to features of the

metabolic syndrome, including NASH [25,26]. The latter findings are confirmed by our study. CXCL10 stimulates monocyte and T-cell migration [27]. Hepatic *CXCL10* mRNA expression is associated with the presence of necro-inflammatory foci in the liver in mice on a methionine- and choline-deficient diet [28]. Our data now add that hepatic *CXCL10* expression is also associated with the development of fibrosis and steatosis in human NASH. However, we showed that this correlation was dependent on morbid obesity and related disorders. Thus, its role in the development of NAFLD needs further investigation in a larger cohort of NAFLD patients, with and without other obesity disorders. IGF1 has a similar function to insulin. IGF1 plasma levels are decreased in individuals with hepatic steatosis [29] and hepatic mRNA expression of *IGF1* is negatively correlated to fibrosis [30]. In line with this, we observed a negative correlation between *IGF1* expression in the liver and steatosis. PAI-1 is known to be an inhibitor of fibrinolysis [31]. Hepatic gene expression and plasma PAI-1 levels are higher in individuals with NAFLD and NASH compared to healthy controls and increase in parallel with the severity of NAFLD features, including lobular inflammation [32]. This was confirmed by our findings of a strong positive correlation between hepatic *PAI-1* expression and lobular inflammation. Inhibition of fibrinolysis by PAI-1 may cause lobular inflammation due to tissue damage induced by increased fibrin deposition.

Interestingly, our study showed that the expression levels of three genes in VAT (*leptin*, *chemerin* and *ANGPT2*) were associated with NAFLD features. This reinforces the detrimental role of VAT in liver diseases [11]. Leptin regulates food intake and energy expenditure and is elevated in obesity [33]. However, the role of *leptin* in the development of NASH has been controversial. Several studies have shown that plasma *leptin* levels are elevated in patients with NASH [34] and are correlated with steatosis [12], whereas others did not find any association [35,36]. The positive association we found between *leptin* expression in VAT and lobular inflammation after adjusting for age, gender, BMI and WHR, suggests that *leptin* contributes to the development of hepatic inflammation in NAFLD. This is in line with the previous findings that leptin is also important in regulating immune function [37]. We observed that the *ANGPT2* expression in VAT is related to lobular inflammation in NASH, although we also showed that this correlation was dependent on the presence of insulin resistance and/or T2D. This is in line with previous research that showed elevated ANPGT2 plasma levels in obese and T2D patients [38-40].

The most striking observation of our study was that *chemerin* expression in VAT was negatively correlated to steatosis, lobular inflammation and portal inflammation

independent of obesity, T2D and HOMA-IR. The literature reports conflicting evidence on the role of chemerin in inflammation and NAFLD. Mice lacking the chemerin receptor, chemokine-like receptor 1, were resistant to central nervous system inflammation [41], but more susceptible to inflammation in models of inflammatory pulmonary disease [42,43]. In addition, these mice were reported have reduced hepatic steatosis and hepatic inflammation in one study [44], whereas this was not affected in another study [45]. Our study now suggests that increased *chemerin* expression in VAT may reduce hepatic inflammation. We further investigated whether this association can be explained by endocrine effects, as adipokines are secreted into the bloodstream from adipose tissue. However, in contrast to earlier studies [46,47], chemerin plasma levels did not correlate with NAFLD features in our study nor with chemerin expression levels in the three tissues under study (Table 3). Further analyses are needed to investigate the underlying mechanisms behind the negative correlation between chemerin expression in VAT and hepatic inflammation.

In conclusion, our results suggest that *leptin*, *chemerin*, *ANGPT2*, *TNF*, *CXCL10*, *IGF1*, *IL1RN* and *PAI-1* could play a role in the development of specific features of the NAFLD phenotype. Most of the observed effects were independent of age, sex, obesity, insulin resistance and type 2 diabetes. As it is not known why some patients with fatty liver progress towards hepatic inflammation while others do not, it is tempting to suggest that adipokines play a role in this process. Further functional studies with gene knock-out animal models and tissue culture experiments may provide more information on the association we have discovered between adipokines and specific features of the NAFLD phenotype.

Acknowledgements

We thank Yanti Slaats for collecting tissue samples and Annemarie van Bijnen for excellent technical assistance. We thank Jan Albert Kuivenhoven and Bart van de Sluis for general support and Jackie Senior for editing the manuscript. This work was funded by the Dutch Diabetes Foundation (grant 2006.00.007; 2009.80.016; 2004.00.018), an IOP genomics grant IGE05012A, the Systems Biology Centre for Energy Metabolism and Aging (SBC-EMA), a Transnational University Limburg grant, two Netherlands Organization for Scientific Research (NWO) VENI grants (863.09.007 to JF and 916.10.135 to LF), a Horizon Breakthrough grant 92519031 from the Netherlands Genomics Initiative (LF),

a Center for Translational Molecular Medicine (www.ctmm.nl) grant, PREDICCt (project grant 01C-104) (DPYK), BBMRI-NL complementary project (project grant CP2013-71).

Conflict of Interest

The authors have no conflicts of interest to declare.

References

1. Cohen JC, Horton JD, Hobbs HH. (2011) Human fatty liver disease: Old questions and new insights. Science 332: 1519-1523.
2. Dixon JB, Bhathal PS, O'Brien PE. (2001) Nonalcoholic fatty liver disease: Predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. Gastroenterology 121: 91-100.
3. Adams LA, Lymp JF, St Sauver J, Sanderson SO, Lindor KD, et al. (2005) The natural history of nonalcoholic fatty liver disease: A population-based cohort study. Gastroenterology 129: 113-121.
4. Adams LA, Sanderson S, Lindor KD, Angulo P. (2005) The histological course of nonalcoholic fatty liver disease: A longitudinal study of 103 patients with sequential liver biopsies. J Hepatol 42: 132-138.
5. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, et al. (1999) Nonalcoholic fatty liver disease: A spectrum of clinical and pathological severity. Gastroenterology 116: 1413-1419.
6. Starley BQ, Calcagno CJ, Harrison SA. (2010) Nonalcoholic fatty liver disease and hepatocellular carcinoma: A weighty connection. Hepatology 51: 1820-1832.
7. Sanyal AJ, American Gastroenterological Association. (2002) AGA technical review on nonalcoholic fatty liver disease. Gastroenterology 123: 1705-1725.
8. Marchesini G, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, et al. (2003) Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. Hepatology 37: 917-923.
9. Tilg H, Moschen AR. (2010) Evolution of inflammation in nonalcoholic fatty liver disease: The multiple parallel hits hypothesis. Hepatology 52: 1836-1846.
10. Wiest R, Moleda L, Farkas S, Scherer M, Kopp A, et al. (2010) Splanchnic concentrations and postprandial release of visceral adipokines. Metabolism 59: 664-670.
11. Hajer GR, van Haeften TW, Visseren FL. (2008) Adipose tissue dysfunction in obesity, diabetes, and vascular diseases. Eur Heart J 29: 2959-2971.
12. Chitturi S, Farrell G, Frost L, Kriketos A, Lin R, et al. (2002) Serum leptin in NASH correlates with hepatic steatosis but not fibrosis: A manifestation of lipotoxicity? Hepatology 36: 403-409.
13. Chamberland JP, Berman RL, Aronis KN, Mantzoros CS. (2013) Chemerin is expressed mainly in pancreas and liver, is regulated by energy deprivation, and lacks day/night variation in humans. Eur J Endocrinol 169: 453-462.
14. Dominguez M, Miquel R, Colmenero J, Moreno M, Garcia-Pagan JC, et al. (2009) Hepatic

expression of CXC chemokines predicts portal hypertension and survival in patients with alcoholic hepatitis. Gastroenterology 136: 1639-1650.

15. Deng Y, Scherer PE. (2010) Adipokines as novel biomarkers and regulators of the metabolic syndrome. Ann N Y Acad Sci 1212: E1-E19.
16. Juge-Aubry CE, Henrichot E, Meier CA. (2005) Adipose tissue: A regulator of inflammation. Best Pract Res Clin Endocrinol Metab 19: 547-566.
17. Wozniak SE, Gee LL, Wachtel MS, Frezza EE. (2009) Adipose tissue: The new endocrine organ? A review article. Dig Dis Sci 54: 1847-1856.
18. Lonardo A, Carani C, Carulli N, Loria P. (2006) 'Endocrine NAFLD' a hormonocentric perspective of nonalcoholic fatty liver disease pathogenesis. J Hepatol 44: 1196-1207.
19. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, et al. (2005) Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 41: 1313-1321.
20. Wolfs MG, Rensen SS, Bruin-Van Dijk EJ, Verdam FJ, Greve JW, et al. (2010) Co-expressed immune and metabolic genes in visceral and subcutaneous adipose tissue from severely obese individuals are associated with plasma HDL and glucose levels: A microarray study. BMC Med Genomics 3: 34.
21. Fu J, Wolfs MG, Deelen P, Westra HJ, Fehrmann RS, et al. (2012) Unraveling the regulatory mechanisms underlying tissue-dependent genetic variation of gene expression. PLoS Genet 8: e1002431.
22. Carter-Kent C, Zein NN, Feldstein AE. (2008) Cytokines in the pathogenesis of fatty liver and disease progression to steatohepatitis: Implications for treatment. Am J Gastroenterol 103: 1036-1042.
23. Aparicio-Vergara M, Hommelberg PP, Schreurs M, Gruben N, Stienstra R, et al. (2013) Tumor necrosis factor receptor 1 gain-of-function mutation aggravates nonalcoholic fatty liver disease but does not cause insulin resistance in a murine model. Hepatology 57: 566-576.
24. Dinarello CA. (2009) Immunological and inflammatory functions of the interleukin-1 family. Annu Rev Immunol 27: 519-550.
25. Pihlajamaki J, Kuulasmaa T, Kaminska D, Simonen M, Karja V, et al. (2012) Serum interleukin 1 receptor antagonist as an independent marker of non-alcoholic steatohepatitis in humans. J Hepatol 56: 663-670.
26. Estep JM, Baranova A, Hossain N, Elariny H, Ankrah K, et al. (2009) Expression of cytokine signaling genes in morbidly obese patients with non-alcoholic steatohepatitis and hepatic fibrosis. Obes Surg 19: 617-624.

27. Taub DD, Lloyd AR, Conlon K, Wang JM, Ortaldo JR, et al. (1993) Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. J Exp Med 177: 1809-1814.
28. Maina V, Sutti S, Locatelli I, Vidali M, Mombello C, et al. (2012) Bias in macrophage activation pattern influences non-alcoholic steatohepatitis (NASH) in mice. Clin Sci (Lond) 122: 545-553.
29. Volzke H, Nauck M, Rettig R, Dorr M, Higham C, et al. (2009) Association between hepatic steatosis and serum IGF1 and IGFBP-3 levels in a population-based sample. Eur J Endocrinol 161: 705-713.
30. Hribal ML, Procopio T, Petta S, Sciacqua A, Grimaudo S, et al. (2013) Insulin-like growth factor-I, inflammatory proteins, and fibrosis in subjects with nonalcoholic fatty liver disease. J Clin Endocrinol Metab 98: E304-8.
31. Cesari M, Pahor M, Incalzi RA. (2010) Plasminogen activator inhibitor-1 (PAI-1): A key factor linking fibrinolysis and age-related subclinical and clinical conditions. Cardiovasc Ther 28: e72-91.
32. Verrijken A, Francque S, Mertens I, Prawitt J, Caron S, et al. (2013) Prothrombotic factors in histologically proven NAFLD and NASH. Hepatology. E-pub ahead of print 23 May 2013; doi: 10.1002/hep.26510.
33. Friedman JM, Halaas JL. (1998) Leptin and the regulation of body weight in mammals. Nature 395: 763-770.
34. Uygun A, Kadayifci A, Yesilova Z, Erdil A, Yaman H, et al. (2000) Serum leptin levels in patients with nonalcoholic steatohepatitis. Am J Gastroenterol 95: 3584-3589.
35. Kashyap SR, Diab DL, Baker AR, Yerian L, Bajaj H, et al. (2009) Triglyceride levels and not adipokine concentrations are closely related to severity of nonalcoholic fatty liver disease in an obesity surgery cohort. Obesity (Silver Spring) 17: 1696-1701.
36. Chalasani N, Crabb DW, Cummings OW, Kwo PY, Asghar A, et al. (2003) Does leptin play a role in the pathogenesis of human nonalcoholic steatohepatitis? Am J Gastroenterol 98: 2771-2776.
37. Carlton ED, Demas GE, French SS. (2012) Leptin, a neuroendocrine mediator of immune responses, inflammation, and sickness behaviors. Horm Behav 62: 272-279.
38. Lieb W, Zachariah JP, Xanthakis V, Safa R, Chen MH, et al. (2010) Clinical and genetic correlates of circulating angiopoietin-2 and soluble tie-2 in the community. Circ Cardiovasc Genet 3: 300-306.
39. Lim HS, Blann AD, Chong AY, Freestone B, Lip GY. (2004) Plasma vascular endothelial growth factor, angiopoietin-1, and angiopoietin-2 in diabetes: Implications for cardiovascular risk and

effects of multifactorial intervention. Diabetes Care 27: 2918-2924.

40. Silha JV, Krsek M, Sucharda P, Murphy LJ. (2005) Angiogenic factors are elevated in overweight and obese individuals. Int J Obes (Lond) 29: 1308-1314.
41. Graham KL, Zabel BA, Loghavi S, Zuniga LA, Ho PP, et al. (2009) Chemokine-like receptor-1 expression by central nervous system-infiltrating leukocytes and involvement in a model of autoimmune demyelinating disease. J Immunol 183: 6717-6723.
42. Bondue B, Vosters O, de Nadai P, Glineur S, De Henau O, et al. (2011) ChemR23 dampens lung inflammation and enhances anti-viral immunity in a mouse model of acute viral pneumonia. PLoS Pathog 7: e1002358.
43. Luangsay S, Wittamer V, Bondue B, De Henau O, Rouger L, et al. (2009) Mouse ChemR23 is expressed in dendritic cell subsets and macrophages, and mediates an anti-inflammatory activity of chemerin in a lung disease model. J Immunol 183: 6489-6499.
44. Ernst MC, Haidl ID, Zuniga LA, Dranse HJ, Rourke JL, et al. (2012) Disruption of the chemokine-like receptor-1 (CMKLR1) gene is associated with reduced adiposity and glucose intolerance. Endocrinology 153: 672-682.
45. Gruben N, Aparicio Vergara M, Kloosterhuis NJ, van der Molen H, Stoelwinder S, et al. (2014) Chemokine-like receptor 1 deficiency does not affect the development of insulin resistance and nonalcoholic fatty liver disease in mice. PLoS One 9: e96345.
46. Kukla M, Zwirska-Korczala K, Hartleb M, Waluga M, Chwist A, et al. (2010) Serum chemerin and vaspin in non-alcoholic fatty liver disease. Scand J Gastroenterol 45: 235-242.
47. Sell H, Divoux A, Poitou C, Basdevant A, Bouillot JL, et al. (2010) Chemerin correlates with markers for fatty liver in morbidly obese patients and strongly decreases after weight loss induced by bariatric surgery. J Clin Endocrinol Metab 95: 2892-2896.

Supplementary information

Supplementary Statistical Notes 1–3

1. Empirical threshold for inter-tissue correlation of adipokines

We performed a permutation analysis to determine the empirical threshold for the inter-tissue correlation of adipokines at a false discovery rate (FDR) of 0.05. For 93 subjects and 69 adipokine probes, we obtained the expression matrix (69 probes x 93 subjects) for each tissue type, allowing missing values for absent samples. We permuted the inter-tissue correlation by randomly reordering the columns of each expression matrix. Then we calculated the Spearman correlation for each probe between the permuted expression matrixes for each probe. This permutation was repeated 1000 times and all *P* values were recorded. Then we determined the FDR at a certain *P* value (P_0). As a result of the permutation, all correlations significant at P_0 level were, by default, false positive. We computed the FDR by dividing the average number of false positive correlations from 1000 permutations by the number of positive correlations detected in the real data and controlled the FDR $\leq$ 0.05 level.

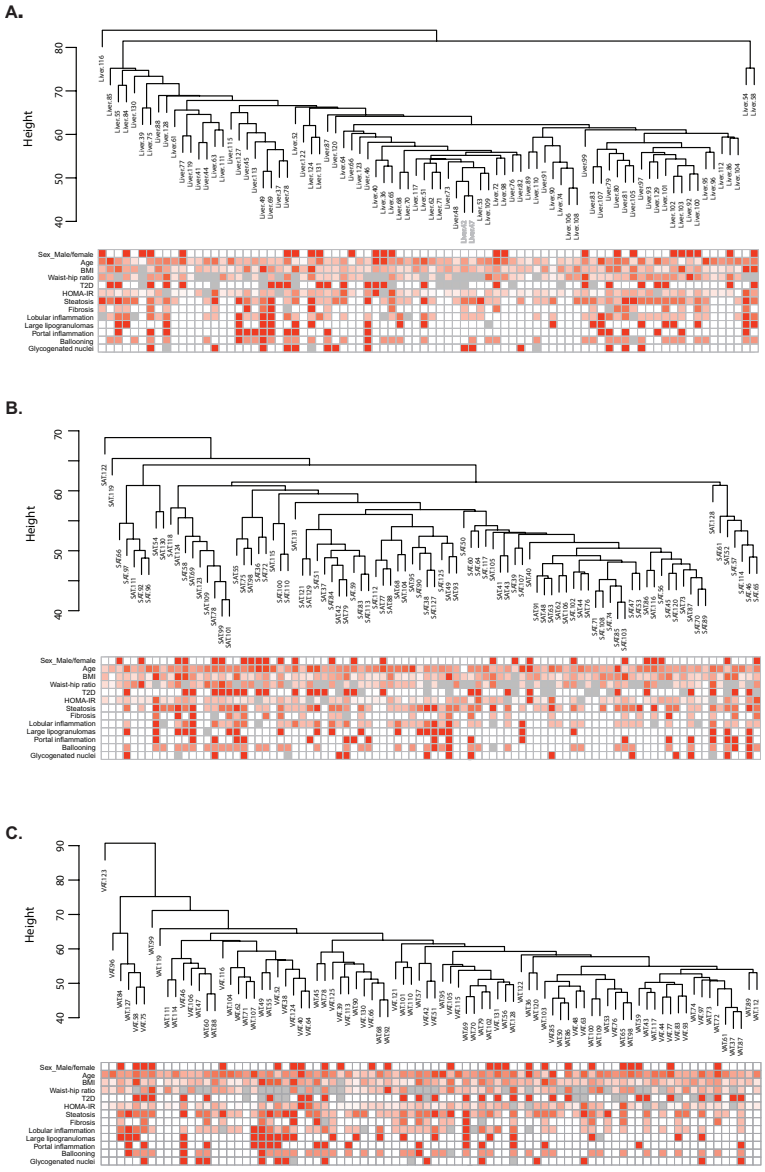
2. Permutation analysis to determine the threshold for the significant correlation between adipokine expression and liver histology

To determine the empirical threshold for the significant correlation between adipokine expression and liver histology, we kept the expression matrix (69 probes x 93 subjects) intact, but permuted the correlation with NAFLD features by shuffling the columns of the NAFLD feature matrix (7 parameters x 93 subjects). Then we computed the Spearman correlations between liver histology scores and the expression levels of adipokines from each tissue type. The permutation was repeated 1000 times and the FDR was controlled at FDR $\leq$ 0.05 level.

3. Regression analysis

To assess whether the correlations between adipokine expression and liver histology were dependent on covariates, we performed a step-wise regression analysis to test the effect of different confounders. First, a conditional analysis was conducted to regress out the effect of age and sex using the model $y_i = age_i + sex_i + e_i$, where y_i, age_i and sex_i refer to the expression value, age and gender of the i^{th} individual. Secondly, we further regressed

out the effect of obesity parameters, including body mass index (BMI) and body mass distribution using waist-to-hip ratio (WHR). The model is described as $y_i = age_i + sex_i + BMI_i + WHR_i + e_i$. Finally, we also regressed out the confounded effect with diabetes and insulin resistance. The model is described as $y_i = age_i + sex_i + BMI_i + WHR_i + T2D_i + IR_i + e_i$. The residuals ($e_i$) of each model were subject to the Spearman correlation analysis with NAFLD features.



SUPPLEMENTARY FIGURE 1. Hierarchical clustering of the individuals based on global gene expression. The hierarchical cluster of individuals based on the expression profiles in (A) liver, (B) SAT and (C) VAT. The heatmaps below the clusters represent the values of sex, age, BMI and seven NAFLD features. The color scheme ranges from white (low score) to red (high score). The grey color indicates a missing value.

SUPPLEMENTARY TABLE 1. Plasma parameters and clinical traits of the study population.

Parameters and traits	Mean (SD)	Min-max
Male/female (#)	26/67	-
Age (years)	44.2 (9.7)	18-67
BMI (kg/ m^2)	46.1 (9.5)	30.7-73.6
WHR	1.0 (0.13)	0.76-1.41
Glucose (mmol/l)	6.45 (1.98)	4.3-14.5
HbA_{1c}	6.54 (1.35)	5.1-12.1
Insulin (mU/l)	19 (10.6)	3.8-53
HOMA-IR	5.42 (3.54)	0.97-18.8
T2D (yes/no)	27 / 53	
Total cholesterol (mmol/l)	5.08 (1.12)	3-9.8
HDL cholesterol (mmol/l)	0.98 (0.37)	0.5-2.8
LDL cholesterol (mmol/l)	3.21 (1)	1.1-7.4
Triglycerides (mmol/l)	2.22 (1.98)	0.63-16.4
NEFA (mmol/l)	0.63 (0.3)	0.12-1.66
ALAT (U/l)	26.5 (16)	6-124
ASAT (U/l)	24.7 (12.4)	7-72
C-reactive protein (mg/l)	10.2 (8.1)	1-37
ANGPT2 (ng/ml)	2.5 (1.1)	1.1-6.7
Chemerin (ng/ml)	84.4 (19.3)	48.1-137.7

Data are represented as means ± standard deviation (SD) and the minimal (min) and maximal (max) values for each features. BMI, body mass index; WHR, waist-to-hip ratio; T2D, type 2 diabetes; HOMA-IR, homeostasis model assessment of insulin resistance NEFA, non-esterified fatty acid; ALAT, alanine aminotransaminase; ASAT, aspartate aminotransaminase; ANGPT2, angiopoietin 2.

SUPPLEMENTARY TABLE 2. The correlation between adipokine expression and NAFLD features in 54 women (age ≤ 50).

Gene	Feature	Tissue	Spearman Corr. (P value)
ANGPT2	Lobular inflam.	VAT	0.54 ($P = 9.8x10^{-5}$)
Leptin	Lobular inflam.	VAT	0.486 ($P = 6.2x10^{-4}$)
	Steatosis	VAT	0.48 ($P = 5.5x10^{-4}$)
Chemerin	Lobular inflam.	VAT	-0.53 ($P = 1.5x10^{-4}$)
	Lobular inflam.	SAT	-0.467 ($P = 5.5x10^{-4}$)
	Portal inflam.	VAT	-0.403 (P = 0.0046)
	Steatosis	VAT	-0.49 ($P = 4.4x10^{-4}$)
IL1RN	Lobular inflam.	Liver	0.49 ($P = 8.9x10^{-4}$)
	Ballooning	SAT	0.37 (P = 0.0070)
CXCL10	Steatosis	Liver	0.32 (P = 0.035)
	Fibrosis	Liver	0.46 (P = 0.0016)
IGF1	Steatosis	Liver	-0.41 (P = 0.0056)
PAI-1	Lobular inflam.	Liver	0.58 ($P = 4.1x10^{-5}$)
	Portal Inflam.	Liver	0.40 (P = 0.0066)
TNF	Lobular Inflam.	Liver	0.36 (P = 0.017)